THE BETA-D-GALACTOSIDASE OF ESCHERICHIA COLI, STRAIN K-12

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The glycolysis of lactose by *Escherichia coli* is of interest for the biochemical problem of disaccharide utilization and as a point of attack for studies of the genetic basis of enzyme constitution. As a basis for further studies in these directions, the present study has been made of a β-D-galactoside-splitting enzyme in cells of strain K-12. This strain was chosen because of its suitability for genetic analysis by recombination methods (Lederberg, 1947).

METHODS

Cell-free extracts. Most preparations were made from cells grown in aerated liquid medium, either a synthetic medium with lactose as the sole carbon source or a complete medium containing 0.1 to 1.0 per cent lactose as well as peptone or tryptic digest of casein. Some preparations were made from cells grown on agar plates. No differences in behavior were apparent between various preparations. The cells were washed with sterile water, by centrifugation and resuspension. Extracts were most conveniently prepared from cells dried *in vacuo* over P_2O_5 at room temperature. Autolysis under benzene and crushing with powdered glass were also successful. Dried cells were triturated and shaken for 2 to 3 hours at 37 C with 50 to 100 volumes of water or buffer. Debris was sedimented at 3,500 rpm for 15 minutes and discarded. The supernatant was usually opalescent but free of bacterial cells or large fragments. Most of the experiments reported here were performed with these crude extracts or with a partially purified fraction with precipitates between 25 and 50 per cent saturation of ammonium sulfate. Substantially the total activity was retained in such fractions, as well as in solutions dialyzed overnight against distilled water.

Galactosidase activity is retained without appreciable loss over periods of several months of storage in the refrigerator, and is unaffected under these conditions by such preservatives as benzene or thymol. The enzyme is precipitated by cold acetone or alcohol, but with appreciable loss of activity, which may be due to the incomplete redispersion of the sediment in aqueous solvent. Cells dried with cold acetone retain considerable activity, little of which is extractable.

Assay with o-nitrophenyl β-D-galactoside (ONPG). A variety of physical, chemical, and biochemical methods have been used for the measurement of galactosidase activity, none of which are entirely satisfactory, particularly for intact cells that glycolyze the split products. The successful application of chromogenic

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substrates, such as *p*-nitrophenyl sulfate for enzyme assay (Huggins and Smith, 1947), suggested that a chromogenic galactoside might be used for assaying galactosidase. Accordingly, *o*-nitrophenyl β-D-galactoside (ONPG) was prepared (Seidman and Link, 1950) and found to be suitable.[3]

The intact glycoside has a negligible optical density at visible wave lengths. Free *o*-nitrophenol (ONP) is, however, capable of a tautomeric change that gives it a yellow color in alkaline solution, with an absorption peak at 420 mμ. ONP is a weak acid, pK 7.3, and the acid, benzenoid tautomer is practically colorless. It must therefore be determined either in well-buffered solutions, in which a fixed proportion of the ONP is dissociated (and colored), or in alkaline solutions, pH 10 or higher, in which a negligible fraction remains undissociated (and colorless).

A Coleman 14 spectrophotometer was used for all photometric assays, with standardized 10-ml sample test tube cuvettes that were found to have an effective optical path of 1.38 cm. In these tubes, a 10^{-5} M solution of alkaline ONP has an optical density of 0.050 (fortuitously integral). The low dilution at which ONP can be accurately measured permits the assay of extremely dilute extracts, equivalent to 10 μg or fewer cells in 10 ml. The activity unit, or "ONPG unit," will be defined as the liberation of ONP at the rate of 10^{-8} moles per minute, corresponding to a galvanometer deflection of 0.1 optical density after 20 minutes.

Unless deliberately modified, the standard procedure for assay was as follows: Samples were made up to 9 ml with M/50 or M/100 sodium phosphate buffer, pH 7.5, and a suitable dilution of cells or extract. After temperature equilibration in a 37 C water bath, initial readings were taken in the spectrophotometer at 420 mμ. One ml M/200 substrate was then added to the tube, and the latter shaken. After 20 minutes of incubation the tubes are read, or for precise measurement the reaction is stopped by the addition of 1 ml molar sodium carbonate. If the latter procedure is used with intact cells, parallel blank runs must be made, because the alkali causes a marked but unstable decrease in the absorbency of the cells. All readings are corrected for dilution by the added solutions and calculated on a 10-ml basis. Bacterial densities were also determined photometrically at the same wave length. An optical density of an intact cell suspension of 0.100 was found to be equivalent to 0.23 mg (dry weight) in 10 ml.

EXPERIMENTAL RESULTS

Kinetics of galactosidase in cell-free extracts. Two desiderata for an enzyme assay procedure are (1) a linear relationship between the quantity of enzyme and the measured product, and (2) a linear time course. These conditions may be achieved when the enzyme is nearly saturated by the concentration of substrate used, or when measurements are made at points where only a small fraction of the substrate has been converted. The linearity of these relationships for the ONPG-galactosidase system under standard conditions is shown in figures 1 and 2.

[3] *p*-Nitrophenyl galactoside should be equally suitable. Aizawa (1939) has noted that this compound is hydrolyzed by emulsin lactase.

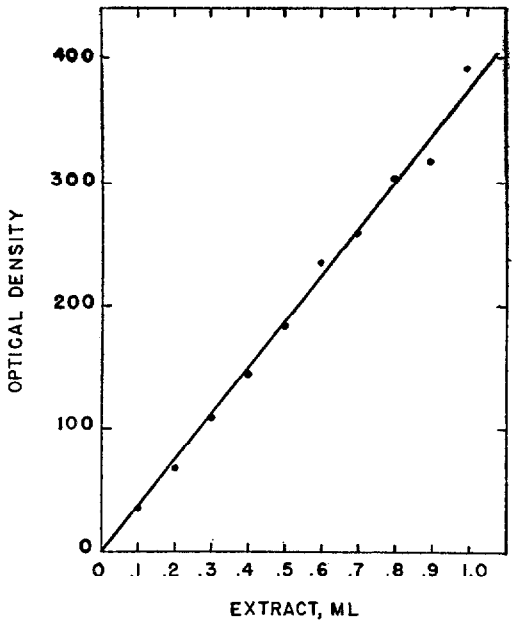

Figure 1. ONPG activity as a function of amount of extract. Standard conditions, using a diluted extract.

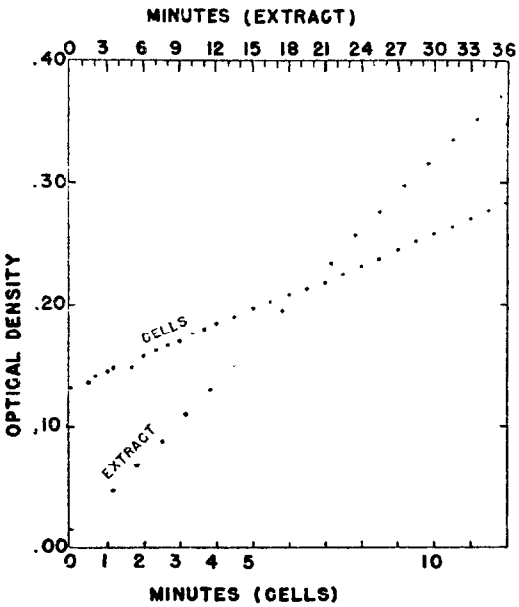

Figure 2. Time course of enzymatic hydrolysis of ONPG. After the sample containing extract or cells had reached approximate temperature equilibrium in the spectrophotometer, ONPG was added at zero time. The ordinate for zero time is corrected for dilution by the added substrate.

A study of the dependence of activity upon ONPG concentration has shown a good agreement between the kinetics of this system and the formulation for a simple enzyme-substrate complex (Wilson, 1949; Lineweaver and Burk, 1934). Kinetic experiments were plotted using Lineweaver and Burk's (1934) reciprocal transformation. When the reciprocal activity is plotted against reciprocal substrate concentration, agreement with theory for a simple enzyme-substrate complex is indicated by the fit of points to a straight line whose ordinate intercept corresponds to the activity of enzyme fully saturated with substrate ("V_{max}").

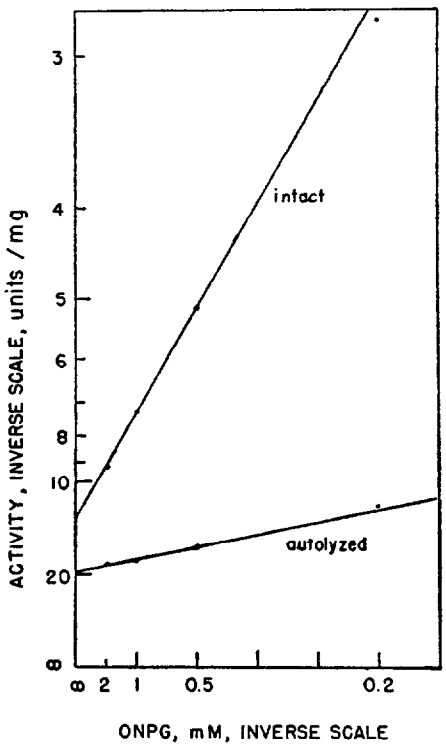

Figure 3. ONPG activity as a function of substrate concentration: autolyzed and intact cells. The recorded activity of the extract has been diminished tenfold in order to make the plots comparable.

The apparent dissociation constant, K_s, was estimated from the slope:intercept ratio by the method of the same authors. K_s (the Michaelis constant) is the substrate concentration at which the enzyme is half-saturated, i.e., activity is half-maximal. All curves were fitted by eye, taking account of a statistical weight of the fourth power of the activity that emphasizes points near the origin (figure 3).

K_s for ONPG has been estimated at 1.3×10^{-4} M in experiments with a number of preparations. This low value is partly responsible for the linear assays under standard conditions.

Extracted galactosidase is profoundly affected by alkali metal ions, tested at

M/50 (figure 4). The extent of activation or inhibition depends on substrate concentration, but cannot be formulated as competitive. In different ionic environments, kinetic experiments give a family of straight lines differing primarily in slope (K_s/V_{max}) but also to some extent in the intercept (V_{max}). This result can be formulated in a number of ways, in particular that interaction of a particular metallic ion with the enzyme protein influences both the absorption of the substrate to the active surface and the rate of decomposition of the complex to form native enzyme and products. This might be expected if the metal ion reacted with groups on the enzyme adjacent to or overlapping the site of substrate binding. This conclusion is not, however, unequivocal.

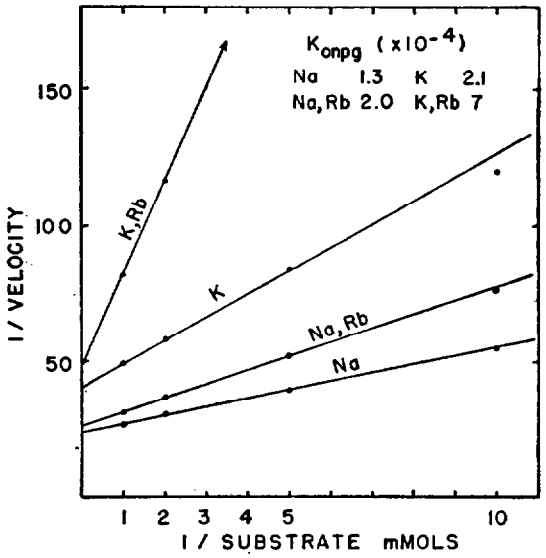

Figure 4. Effects of metal ions on ONPG activity. The indicated metals were added as M/50 chloride to a system made up in M/100 potassium phosphate buffer. Other conditions standard, with fixed amount of diluted extract in each tube.

With activity in dilute potassium buffer as a reference, the effects of different ions can be summarized as follows: Increasing K^+ from M/500 to M/10 has practically no effect. Li^+, NH_4^+, and Cs^+, M/50, are also substantially inert. Na^+, however, stimulates apparent activity with a full effect at about M/50, but Rb^+ is inhibitory. Inhibition by Rb^+ is reversed either by Na^+ or K^+, suggesting that the various ions compete with each other, and possibly with H^+, to account for the effect of varying Na^+ alone. This effect has not yet been adequately studied, and should be investigated with purified preparations rather than crude extracts. Although, as will be considered later, the effect of Na probably has little or no physiological significance, it presents a nearly unique example of an enzymic function of Na^+ in a simple system. The potentialities of this system for the study of simple competitive behavior are extended by the fact that a number

of substituted ammonium ions such as ethanolammonium and ethylenediammonium behave, like rubidium, as inhibitors.

No effect of divalent cations or of anions—chloride, sulfate, nitrate, acetate, and phosphate—was found. Particular attention was paid to phosphate because of the possibility that galactosidase might be phosphorolytic. However, no effect of phosphate was noted on activity in glycerophosphate or in barbital buffer, where direct analysis for inorganic phosphate showed the latter to be present at less than 10^{-6} molar. However, direct proof that galactose, per se, is a product of the reaction has not yet been secured.

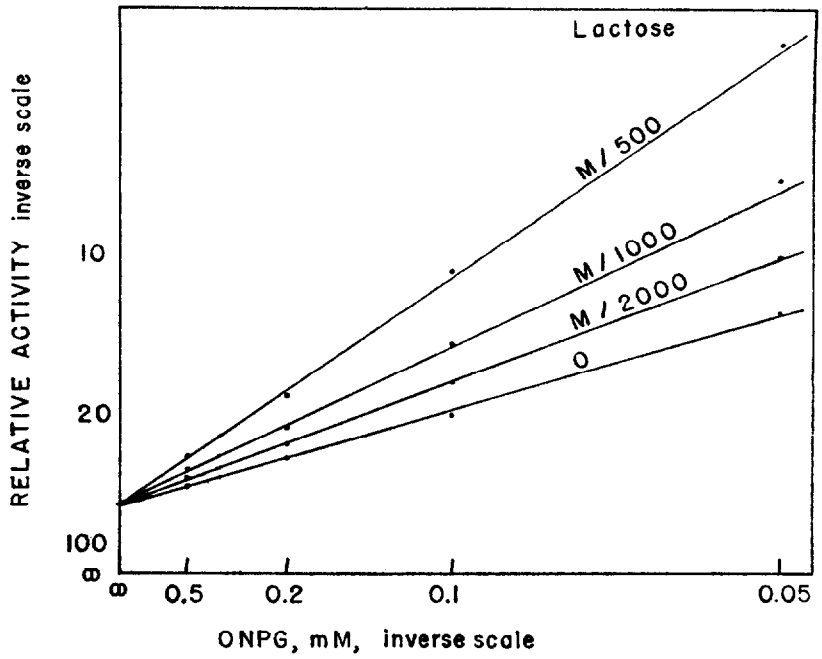

Figure 5. Kinetics of inhibition of ONPG activity by lactose. Standard conditions were used with the addition of the quantity of lactose indicated on the curves. The common intercept is extrapolated.

Competitive inhibition. The specificity of glycosidases from various sources has been found to relate primarily to the glycosidic residue of the substrate, the aglycone usually having only a secondary effect (Pigman, 1944). Although the colorimetric method does not permit a test of the efficiency of splitting of other galactosides, their affinity for the enzyme can be determined by treating them as competitive inhibitors. That is, the binding function of alternative substrates for galactosidase can be evaluated from the kinetics of inhibition of ONPG activity. Such inhibition is expected if the alternative substrate competes with ONPG for attachment to the active enzyme surface.

The kinetic behavior of lactose as a competitive substrate is shown in figure

5. The Michaelis constant K_i for lactose, or other alternative substrate, can be calculated from the expression (from Lineweaver and Burk, 1934):

$$1/V = 1/V_{max} (1 + [1 + I/K_i] K_s/S)$$

where S and I are the concentrations of ONPG and lactose, respectively. The value of K_i is obtained from the increase in slope, K_sI/K_i, of the $1/V$ vs. $1/S$ plots at a given inhibitor concentration, I. Competitive inhibition is indicated in such plots by a family of straight lines with a common ordinate intercept (V_{max}), but with slopes dependent on the inhibitor concentration. Lactose (figure

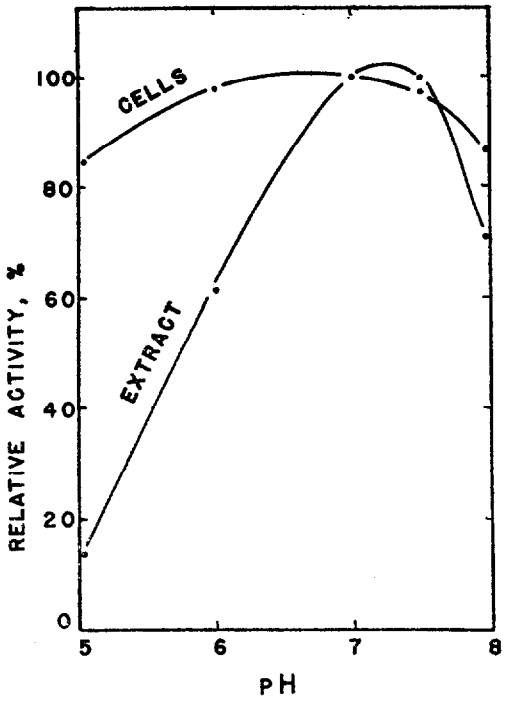

Figure 6. pH response of ONPG activity of extract and of intact cells. Sodium phosphate buffer M/50 was used at each pH. Other conditions were standard. The activity units are arbitrary and are adjusted for unit activity at pH 7.

5) and other galactoside substrates have been found to follow this type of inhibition. K values obtained from such plots with a number of galactosides are enumerated in table 1.

In contrast to the competitive inhibition shown by galactoside analogues, glucose and other reducing sugars show a substantially noncompetitive inhibition at concentrations of M/10 or higher. This effect has not been studied in detail.

Nonspecific inhibitors. Other inhibitors were tested by adding them to the test system prior to adding substrate. Cu^{++} and Hg^{++} salts, M/1,000, immediately inactivated the enzyme. Iodoacetate was relatively innocuous, M/200 giving 20

per cent inhibition. Sodium fluoride in phosphate buffer inhibited 40 per cent at M/500 and over 90 per cent at M/100. In barbital buffer, fluoride had no effect, but became inhibitory if phosphate was added. The addition of Mg^{++}, M/200, aggravated fluoride-phosphate inhibition although it had no effect in the absence of fluoride (cf. Warburg and Christian, 1942). Although this over-all behavior resembles that of magnesium protein enzymes, further study properly demands highly purified preparations.

Intracellular galactosidase and adaptation. The activity and kinetics of galactosidase in intact cells are strikingly different from those of disrupted cells or cell-free extracts. As seen in figure 3, in one experiment, the extrapolated V_{max} of the cellular enzyme was 12.3 ONPG units per mg cells (dry weight), whereas benzene-autolyzed aliquots showed an activity equivalent to 199 units per mg. In addition, the kinetics of the intact cells, though still apparently linear, show a K_s of 6.3×10^{-4} molar, about five times higher than the extract, so that the discrepancy in apparent activity is magnified at lower substrate concentrations.

TABLE 1

*Dissociation constants of β-galactosidase with β-galactosides**

COMPOUND	K_s	COMPOUND	K_s
ONPG	1.3×10^{-4} M	Methyl β-galactoside	1.3×10^{-2}
Lactose	1.4×10^{-3}	*n*-Butyl β-galactoside	5.1×10^{-4}
Galactose	2.1×10^{-2}	Lactitol	5.1×10^{-3}
Ca-lactobionate	4.7×10^{-2}		

* The value for Ca-lactobionate was obtained by the inhibition of ONPG activity at a single ONPG concentration. All other values were obtained from the plots of reciprocal ONPG activity vs. reciprocal ONPG concentration at one or more values of inhibitor concentration.

Similar differences are found between intact cell suspensions and measured aliquots treated with thymol or dried *in vacuo*.

Other treatments are capable of "activating" galactosidase, including storage of the cells in M/10 buffer. Clearly, considerable caution must be exercised to preserve cell suspensions in native condition. Storage in distilled water at refrigerator temperatures appears, however, to be innocuous.

Intact cells show other distinctive responses, in addition to the difference in kinetic properties from the extracted enzyme. The response to pH of the medium is much less abrupt for intact cells. The pH optimum for extract activity is approximately 7.3. That for intact cells may be shifted to a somewhat lower pH, but the flatness of the plateau makes an accurate determination difficult (figure 6). This effect suggests that the internal milieu is not closely concordant with the composition of the exterior. Similarly, Rb^+ and Na^+ ions, which influence the activities of extracts, are essentially inert to intact cells. It may be assumed that the kinetic behavior of intact cells toward the substrate is based on similar restrictions on the transport of the substrate to the enzyme within the

cells. Similar findings have been reported by Myrbäck and Vasseur (1943) and others working with yeast glycosidases.

Beginning with Karström (1930), many workers have observed that lactatic activity of *E. coli* cells was dependent on their prior exposure to lactose, preferably under conditions of growth. That is to say, the lactase of *E. coli* is adaptive. Adaptation to lactose has been regarded as a substrate-induced synthesis of enzyme protein, although small amounts of lactase might persist in unadapted cells (review by Monod, 1947).

This conclusion was challenged by Deere (1939), who observed that lytic treatments of unadapted cells "activated" their lactase to a marked extent. He concluded that adaptation might be comparable to lytic activation, viz., as some sort of permeability effect. He came to a similar conclusion concerning the activity of lactase in the genetically differentiated cells of a lactose-negative culture and its lactose-positive mutants. Unfortunately, Deere was unable, for technical reasons, to make an adequate test for the lytic activation of adapted cells. In

TABLE 2

Lytic activation of adapted and unadapted Escherichia coli

GROWTH SUBSTRATE	ONPG ACTIVITY, UNITS PER MG	
	Intact cells	Benzene-treated cells
Lactose + peptone	15a; 14b; 6.4c	136a; 297b; 300c
Maltose + peptone	2.6a; 0.9b; 0.1c	58a; 22b; 1.8c
Glucose + peptone	0.1±a	8.1a
Peptone	0.2c	2.3c
Lactose	12d	297d
Maltose	0+d	0.3d
Glucose	0+d	0.3d
Sodium succinate	0.4d	1.7d

Letters following the figures in the table refer to different experiments.

E. coli K-12, the ONPG activities of adapted and unadapted cells are both activated by a similar factor by lytic treatment, such as autolysis under benzene (table 2). It may be concluded that there is a real difference in the enzyme content of these cells, at least with respect to lytic "activation."

A single experiment was conducted to verify lytic activation with respect to lactose and to determine the lactatic equivalent of an ONPG unit. Intact cells were studied manometrically, at 32 C in M/20 bicarbonate and 100 per cent CO_2 buffer. With 10 mg lactose per 2 ml cell suspension, a $Q_{CO_2}^{NaHCO_3} = 34$ was obtained for adapted cells, corresponding to the glycolysis of 5.1 mμM lactose per minute per mg (dry weight). This value is obtained by assuming the formation of 2.5 equivalents of acid per mole of hexose glycolyzed (Stokes, 1949). Q = 40 for glucose under comparable conditions in the same experiment, and monose was not accumulated by the intact cells, so that the rate of splitting of lactose may be the limiting factor in lactose fermentation. The V_{max} for the intact cells, measured with ONPG, was 7.5 units per mg.

An aliquot of the cell suspension was shaken with benzene for 3 to 4 hours. The autolyzed cells showed an activity (V_{max}) of 143 ONPG units per mg. Splitting of lactose was measured by a modification of Barfoed's method for monoses (Caputto, Leloir, and Trucco, 1948), and in a 1 per cent lactose solution it was catalyzed at the rate of 2.1×10^{-7} moles per minute per mg cells (dry weight). We may compute the lactatic equivalent of one ONPG unit as 1/143 this figure, or 0.15×10^{-8} moles of lactose per minute. This is only 15 per cent of the rate at which ONPG is decomposed per unit.

A comparison of these rates for the intact and disrupted cells shows a 19-fold increase in ONPG activity with lysis and approximately a 41-fold increase in lactose-splitting activity. The discrepancy is not necessarily a real one, although it may be: K_s for lactose with intact cells was not established; the manometric assay of the intact cells was conducted at a lower temperature (32 C) than was used for lactose hydrolysis by the autolyzate (37 C); and the precision of the chemical method leaves much to be desired. However, there can be no doubt that the activity for lactose is activated by lytic treatment along the same lines as ONPG activity.

The conclusion that unadapted cells are completely deficient in lactase is probably not warranted. Manometric experiments would usually miss activity of the order of a small per cent of adapted cells, or such slight activity might be misinterpreted as a gradual adaptation in the reaction vessel. In addition, the criterion for adaptation has usually been a difference between cells grown on glucose as against lactose. Glucose-grown cells have the lowest residual ONPG activity of any unadapted cells tested. Cells grown on nonglucose substrates may have considerable ONPG activity (table 2). The depressing effect of glucose may be related to its competitive behavior in diauxic growth (Monod, 1947) and in enzymatic adaptations in yeast (Spiegelman, 1946).

DISCUSSION

Since the data of the present paper are preliminary to work in progress on the genetic control (Lederberg, 1948) and adaptive formation of β-D-galactosidase in *E. coli*, a detailed discussion at this point is unwarranted. Several of the problems of the enzyme chemistry of galactosidase, in particular the interactions with metal ions, require the use of preparations more highly purified than can be readily accomplished with bacterial enzymes. However, the durability to storage and preparative reagents shown by galactosidase points to the possibility of such purification.

The author has purposely refrained from using the term "lactase" in connection with the ONPG-splitting enzyme. Although no contrary evidence is now apparent, direct proof is lacking for the participation of the ONPG-ase as the sole pathway of lactose utilization. This caution is especially apt in view of the anomalous behavior of *Saccharomyces fragilis* (Caputto, Leloir, and Trucco, 1948) and of *Lactobacillus bulgaricus* (Snell *et al.*, 1948) toward lactose; although the latter organism possesses an active ONPG-ase, uncomplicated hydrolysis of lactose cannot account for its ability to utilize lactose under conditions such

that the hydrolytic products are unavailable. In addition, the analysis of maltose fermentation by *E. coli* K-12 mutants has revealed previously unsuspected mechanisms whereby free hexose is by-passed (Doudoroff *et al.*, 1949; Monod and Torriani, 1950).

This study also poses a now familiar question: how to correlate the behavior of extracted enzymes with their intracellular economy. Galactosidase provides a simple illustration of the perturbations induced by disrupting the structure of the cell, perhaps comparable in some respects to the transformation of "cyclophorase" of animal tissues which is induced by comparable lytic procedures (Huennekens, 1951).

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SUMMARY

β-D-Galactosidase (lactase?) was extracted from adapted cells of *Escherichia coli*, strain K-12. A method of assay was developed based upon the colorimetric determination of nitrophenol released from a new chromogenic substrate, *o*-nitrophenyl β-D-galactoside. The kinetics of hydrolysis of this substrate were studied, indicating a close fit to the Michaelis theory. Alkali metal ions were found to influence both the efficiency and the affinities on the enzyme. Other galactoside substrates, including lactose, methyl, *n*-butyl, and sorbityl analogues, lactobionic acid, and galactose itself, were found to combine with the enzyme, and could be treated as competitive inhibitors. The dissociation constants for each of these compounds was determined.

The apparent galactosidase activity of intact cells was greatly "activated" by drying and autolytic procedures, accounting for Deere's (1939) conclusion that adaptation and genetic competence were based upon permeability differences. Small amounts of the enzyme were found in unadapted cells.

Intracellular enzyme also differed from extracts in kinetic behavior, pH response, and response to alkali metals, suggesting an active regulation of the *milieu intérieur* of these cells.

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